

## Studies *in vivo* and *in vitro* on the uptake and degradation of soluble collagen $\alpha 1(I)$ chains in rat liver endothelial and Kupffer cells

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Intravenously administered  $^{125}\text{I}$ -labelled monomeric  $\alpha 1$  chains ( $^{125}\text{I}-\alpha 1$ ) of collagen type I were rapidly cleared and degraded by the liver of rats. Isolation of the liver cells after injection of the label revealed that the uptake per liver endothelial cell equalled the uptake per Kupffer cell, whereas the amount taken up per hepatocyte was negligible. The uptake of  $^{125}\text{I}-\alpha 1$  in cultured cells was 10 times higher per liver endothelial cell than per Kupffer cell. The ligand was efficiently degraded by cultures of both cell types. However, spent medium from cultures of Kupffer cells, unlike that from cultures of other cells, contained gelatinolytic activity which degraded  $^{125}\text{I}-\alpha 1$ . The presence of hyaluronic acid, chondroitin sulphate or mannose/*N*-acetylglucosamine-terminal glycoproteins, which are endocytosed by the liver endothelial cells via specific receptors, did not interfere with binding, uptake or degradation of  $^{125}\text{I}-\alpha 1$  by these cells. Unlabelled  $\alpha 1$  and heat-denatured collagen inhibited the binding to a much greater extent than did native collagen. The presence of fibronectin or  $\text{F(ab')}_2$  fragments of anti-fibronectin antibodies did not affect the interaction of the liver endothelial cells, or of other types of liver cells, with  $^{125}\text{I}-\alpha 1$ . The accumulation of fluorescein-labelled heat-denatured collagen in vesicles of cultured liver endothelial cells is evidence that the protein is internalized. Moreover, chloroquine, 5-dimethylaminonaphthalene-1-sulphonylcadaverine (dansylcadaverine), monensin and cytochalasin B, which impede one or more steps of the endocytic process, inhibited the uptake of  $^{125}\text{I}-\alpha 1$  by the liver endothelial cells. Leupeptin, an inhibitor of cathepsin B and 'collagenolytic cathepsins', inhibited the intralysosomal degradation of  $^{125}\text{I}-\alpha 1$ , but had no effect on the rate of uptake of the ligand. The current data are interpreted as follows. (1) The ability of the liver endothelial cells and the Kupffer cells to sequester circulating  $^{125}\text{I}-\alpha 1$  efficiently may indicate a physiological pathway for the breakdown of connective-tissue collagen. (2) The liver endothelial cells express receptors that specifically recognize and mediate the endocytosis of collagen  $\alpha 1(I)$  monomers. (3) The receptors also recognize denatured collagen (gelatin). (4) Fibronectin is not involved in the binding of  $\alpha 1$  to the receptors. (5) Degradation occurs intralysosomally by leupeptin-inhibitable cathepsins.

The most abundant vertebrate protein, collagen, constitutes an important structural basis for the connective tissue. The turnover of most collagens is rather slow (Robins, 1982). For instance, Nissen *et al.* (1978) reported a half-life of 60 days for skin collagen in mature rats. Nevertheless, the fact that the protein exists in such great quantity suggests

that, under normal conditions, there must be a significant overall catabolism of collagen. Moreover, physiological processes such as growth, development, morphogenesis, remodelling and repair, as well as pathophysiological conditions such as burns and mechanical or inflammatory tissue traumas, are correlated with an increased degradation of collagen. Yet the catabolic pathways of this proteins are poorly understood (Gross, 1981).

Abbreviations used: dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

Vertebrate pro-collagenase, which is attached to the collagen fibres in normal connective tissue (Vater *et al.*, 1978), may, on activation, cleave collagen into two pieces (Bornstein & Traub, 1979). This reaction has been suggested as the initial step in the physiological degradation of collagen. In various types of tissue traumas, large collagen-containing fragments of connective tissue as well as soluble collagen peptides may be released. Soluble and particulate collagen may be taken up and degraded by macrophages (Hopper *et al.*, 1976; Tonaki *et al.*, 1976). However, under normal conditions, and even more so in tissue traumas, it is likely that collagen enters the general circulation where it may disturb haemostasis, e.g. by inducing coagulation by interaction with platelets (Barnes, 1982). Therefore, an efficient clearance of collagen from the circulation is of critical importance.

In the present study we have investigated the fate of monomeric collagen  $\alpha 1(I)$  peptides administered intravenously into rats. After identification of the liver endothelial cells as the major site of uptake *in vivo*, receptor-mediated endocytosis with subsequent intralysosomal degradation of the ligand was demonstrated in cultivated liver endothelial cells.

## Materials and methods

### Chemicals and animals

Bovine serum albumin (fraction V), chloroquine, bacterial collagenase (type V) for isolation of liver cells, cytochalasin B, dansylcadaverine, leupeptin, monensin and ovalbumin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Proteinase-free bacterial collagenase for analytical cleavage of  $\alpha 1$  was from Advance Biofacture Corp., Lynbrook, NY, U.S.A. Purified rat monomeric  $\alpha 1(I)$  was kindly given by Dr. S. Gay, University of Alabama in Birmingham, AL, U.S.A. Neutral-salt-soluble collagen from rat tail, and chondroitin sulphate from bovine nasal cartilage were kindly donated by Dr. Å. Wasteson, University of Uppsala, Uppsala, Sweden. Fibronectin was purified according to Vuento & Vaheri (1979). The cell-binding fragment of fibronectin was isolated as described by Johansson (1985). Laminin was kindly donated by Dr. R. Timpl, Max-Planck-Institut, Martinsried, Federal Republic of Germany. Percoll and hyaluronic acid were from Pharmacia, Uppsala, Sweden. Culture medium (Iscove's medium) and the antibiotics Gentamicin and Fungizone were from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. Fluorescein isothiocyanate was obtained from Fluka AG, Buchs, Switzerland. Na<sup>125</sup>I was purchased from The Radiochemical Centre, Amer-

sham, Bucks., U.K. Male Sprague-Dawley rats, weighing approx. 200g, were from Anticimex, Stockholm, Sweden.

### Radiolabelling of $\alpha 1$

Labelling of  $\alpha 1$  and fibronectin with <sup>125</sup>I was performed by the chloramine-T method using Iodobeads (Pierce Chemical Co., Rockford, IL, U.S.A.). The resulting preparations of <sup>125</sup>I- $\alpha 1(I)$  and <sup>125</sup>I-fibronectin had specific radioactivities of  $2.2 \times 10^6$  c.p.m./ $\mu$ g and  $6 \times 10^6$  c.p.m./ $\mu$ g respectively. <sup>125</sup>I radioactivity was determined in a Packard 5260 Auto-Gamma scintillation spectrometer (Packard Instrument Co., Downers Grove, IL, U.S.A.).

### Labelling of collagen with fluorescein isothiocyanate

Heat-denatured (30 min at 60°C) neutral-salt-soluble collagen (20 mg/ml) was incubated with fluorescein isothiocyanate (50  $\mu$ g/ml) in 0.1 M-carbonate/bicarbonate buffer, pH 9, at 4°C for 18 h. Unchanged dye was removed by gel filtration through a PD-10 column (Pharmacia Fine Chemicals) eluted with phosphate-buffered saline (Smedsrød *et al.*, 1985). Sterile filtered fluorescein-labelled collagen was stored at 4°C.

### Isolation and cultivation of liver cells

The procedure for isolation of liver cells without the use of Pronase has been described in detail by Smedsrød & Pertoft (1985). Briefly, the rat liver is dispersed by perfusion with collagenase, and the resulting single-cell suspension is centrifuged at low speed to sediment the hepatocytes selectively. After resuspension and density centrifugation of the cells in the pellet the hepatocytes are purified to nearly 100%. Monolayer cultures of these cells are established and maintained on supports coated with fibronectin, fibronectin cell-binding fragment or laminin (5  $\mu$ g of protein added per cm<sup>2</sup> growth area). The supernatant from the low-speed centrifugation is enriched in sinusoidal cells. After a further purification of these cells by density-gradient centrifugation to get rid of cell debris, hepatocytes and erythrocytes, the cells are further purified by selective substrate adherence. Thus pure monolayer cultures of Kupffer cells are established on glass or plastic, whereas pure monolayer cultures of liver endothelial cells are established on the types of substrates that were described above for the cultivation of hepatocytes. The Kupffer cells were identified by their specific ability to bind erythrocytes coated with IgM and inactivated complement component C3b, whereas the liver endothelial cells were identified by their specific ability to accumulate fluorescein-labelled ovalbumin (Smedsrød *et al.*, 1982). The hepatocytes, which are much larger than other types of

liver cells, were identified by size. The cultures obtained show less than 5% cross-contamination. Because collagen has affinity for fibronectin, but not for fibronectin cell-binding fragment or laminin (Sakashita *et al.*, 1980; Johansson & Höök, 1984), the latter two proteins were preferred as growth substrates in the present study. The cells were seeded and grown in Costar dishes (Costar, Cambridge, MA, U.S.A.; growth areas 2 and 9.6 cm<sup>2</sup> respectively) in Iscove's medium containing Gentamicin (200 µg/ml) and Fungizone (50 µg/ml). The cells were cultured at the following densities: Kupffer cells, 70 000 cells/cm<sup>2</sup>; liver endothelial cells, 200 000 cells/cm<sup>2</sup>; and hepatocytes, 100 000 cells/cm<sup>2</sup>. At 2 h after seeding, the cultures were washed extensively with phosphate-buffered saline, and immediately thereafter the experiments were initiated.

#### *Uptake and degradation of <sup>125</sup>I-α1 in vivo*

Immediately after the injection of <sup>125</sup>I-α1 [6.5 µg (13 × 10<sup>6</sup> c.p.m.) in 1 ml of phosphate-buffered saline] through the tail vein, the monitoring of the blood radioactivity was started by taking blood samples (50 µl) from the tail tip with calibrated capillary tubes. The blood samples were quickly mixed with 0.5 ml of 20% trichloroacetic acid to precipitate non-degraded <sup>125</sup>I-α1. After centrifugation of the precipitates the pellet and the supernatant respectively were analysed for radioactivity. The uptake of <sup>125</sup>I-α1 by the different organs was determined as follows. The liver content of <sup>125</sup>I was assessed after a rapid perfusion through the portal vein with 0.9% NaCl (4°C) to wash out unbound label. The amount of <sup>125</sup>I-α1 taken up by the spleen was determined by subtracting the radioactivity of the spleen blood [170 µl of blood per g of spleen (Dittmer, 1961)] from the measured spleen radioactivity. The total radioactivity in blood was calculated by using the knowledge that rats contain 5.75 ml of blood per 100 g body weight (Dittmer, 1961).

In order to determine the uptake of <sup>125</sup>I-α1 *in vivo* in the various populations of liver cells, a dose of 9 µg (18 × 10<sup>6</sup> c.p.m.) of <sup>125</sup>I-α1 in 1 ml of phosphate-buffered saline was administered intravenously, and after 10 min the cells were isolated as described by Smedsrød & Pertoft (1985) (see also Scheme 1 below).

#### *Binding, accumulation and degradation of <sup>125</sup>I-α1 by cultivated liver cells*

Cells were seeded as described above and were then incubated in medium containing <sup>125</sup>I-α1 (0.2 nM) and various other ligands or drugs. All incubations were carried out with 1% bovine serum albumin in the absence of serum. The total

incubation volumes were 250 µl (2 cm<sup>2</sup> growth area) or 600 µl (9.6 cm<sup>2</sup> growth area). Time and temperature of incubation of the various experiments are specified in the Results section. Cell-associated radioactivity was determined after washing the cultures four times with phosphate-buffered saline (4°C) by dissolving the cell layer in 1% sodium dodecyl sulphate and 0.3 M-NaOH, and measuring the <sup>125</sup>I radioactivity. <sup>125</sup>I-labelled material in the culture medium that did not precipitate with an equal volume of 20% trichloroacetic acid was taken as degraded α1-chains.

#### *Accumulation of fluorescein-labelled collagen into liver endothelial cells*

Liver endothelial cells were cultured under the conditions described above in the presence of 0.1 mg of fluorescein-labelled heat-denatured neutral-salt-soluble collagen/ml. The experiment was terminated after 1 h. The cells were washed five times with phosphate-buffered saline before fixation and mounted for examination by fluorescence microscopy as described by Smedsrød *et al.* (1982). Photomicrographs were taken with a Kodak Tri-X film.

#### *Preparation of antibodies against fibronectin*

Antibodies to fibronectin were prepared as described by Johansson & Höök (1984). Briefly, rabbits were immunized with rat fibronectin, and IgG antibodies were purified by binding to protein A coupled to Sepharose 4B (Pharmacia Fine Chemicals). Bivalent antigen-binding fragments [F(ab')<sub>2</sub>] were prepared by digestion of anti-(rat fibronectin) IgG (50 mg/ml) with pepsin (1 mg/ml) in 0.1 M-acetate buffer, pH 4.5, at 37°C for 16 h. After neutralization and dialysis against phosphate-buffered saline, the digest was chromatographed on a column of protein A-Sepharose to remove intact IgG molecules and Fc fragments. The F(ab')<sub>2</sub> fragments were affinity-purified by chromatography on rat fibronectin conjugated with Sepharose CL-4B eluted with 50 mM-citrate buffer, pH 3.0, and finally dialysed against phosphate-buffered saline.

## **Results**

#### *Fate of circulating <sup>125</sup>I-α1*

Fig. 1 demonstrates that most of the intravenously administered <sup>125</sup>I-α1 was cleared from the circulation within 2–3 min. Degradation products started to appear in the blood after approx. 10 min. The concentration in the blood of acid-soluble degradation products reached a peak at about 1 h after the injection, and then started to decline.

Analysis of the radioactivity in various organs after administration of the ligand revealed that the

liver is the major site of uptake (Table 1). Isolation of the liver cells after injection of  $^{125}\text{I}$ - $\alpha 1$  disclosed that the ligand was taken up mainly by the Kupffer cells and the liver endothelial cells, whereas the hepatocytes accounted for a negligible uptake (Scheme 1). The uptake per cell was similar in the Kupffer cells and the liver endothelial cells.

#### Interaction of $^{125}\text{I}$ - $\alpha 1$ with isolated liver cells

Experiments with pure cultures of various types of liver cells demonstrated that the liver endothelial cells, and to a lesser extent the Kupffer cells, took up  $^{125}\text{I}$ - $\alpha 1$  (Table 2). Cultured hepatocytes did not bind or degrade the label (results not shown). After 30 min of incubation at  $37^\circ\text{C}$  the proportions of ligand degraded in the cultures of Kupffer cells

and liver endothelial cells were 70 and 30% respectively. Uptake and degradation were unaffected by the addition of fibronectin. The high rate of degradation in the Kupffer-cell cultures, along with the very low uptake by the cells, suggested that the breakdown of  $^{125}\text{I}$ - $\alpha 1$  in these cultures might have occurred extracellularly by proteinases secreted by the Kupffer cells. To test this possibility, spent media from cultures of Kupffer cells and

Table 1. Fate of circulating  $^{125}\text{I}$ - $\alpha 1$   
A rat was injected intravenously with  $6.5\text{ }\mu\text{g}$  of  $^{125}\text{I}$ - $\alpha 1$  ( $13 \times 10^6$  c.p.m.) and killed 10 min later. Blood was collected, and the listed organs were dissected out. Radioactivity was measured as described in the Materials and methods section.

Organ	Radioactivity (% of injected dose)
Blood	9.5
Liver	61
Spleen	1.1
Kidneys	9.6
Lungs	0.3
Heart	0.2

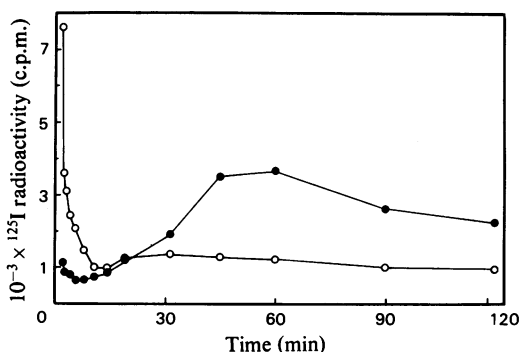


Fig. 1. Clearance and degradation of intravenously administered  $^{125}\text{I}$ - $\alpha 1$

A dose of  $6.5\text{ }\mu\text{g}$  of  $^{125}\text{I}$ - $\alpha 1$  was injected into a rat, and the variation with time of the blood content of undegraded (acid-precipitable, open symbols) and degraded (acid-soluble, filled symbols) ligand was recorded. Injection of  $^{125}\text{I}$ - $\alpha 1$ , sampling of the blood and analysis of intact and degraded ligand were as described in the Materials and methods section.

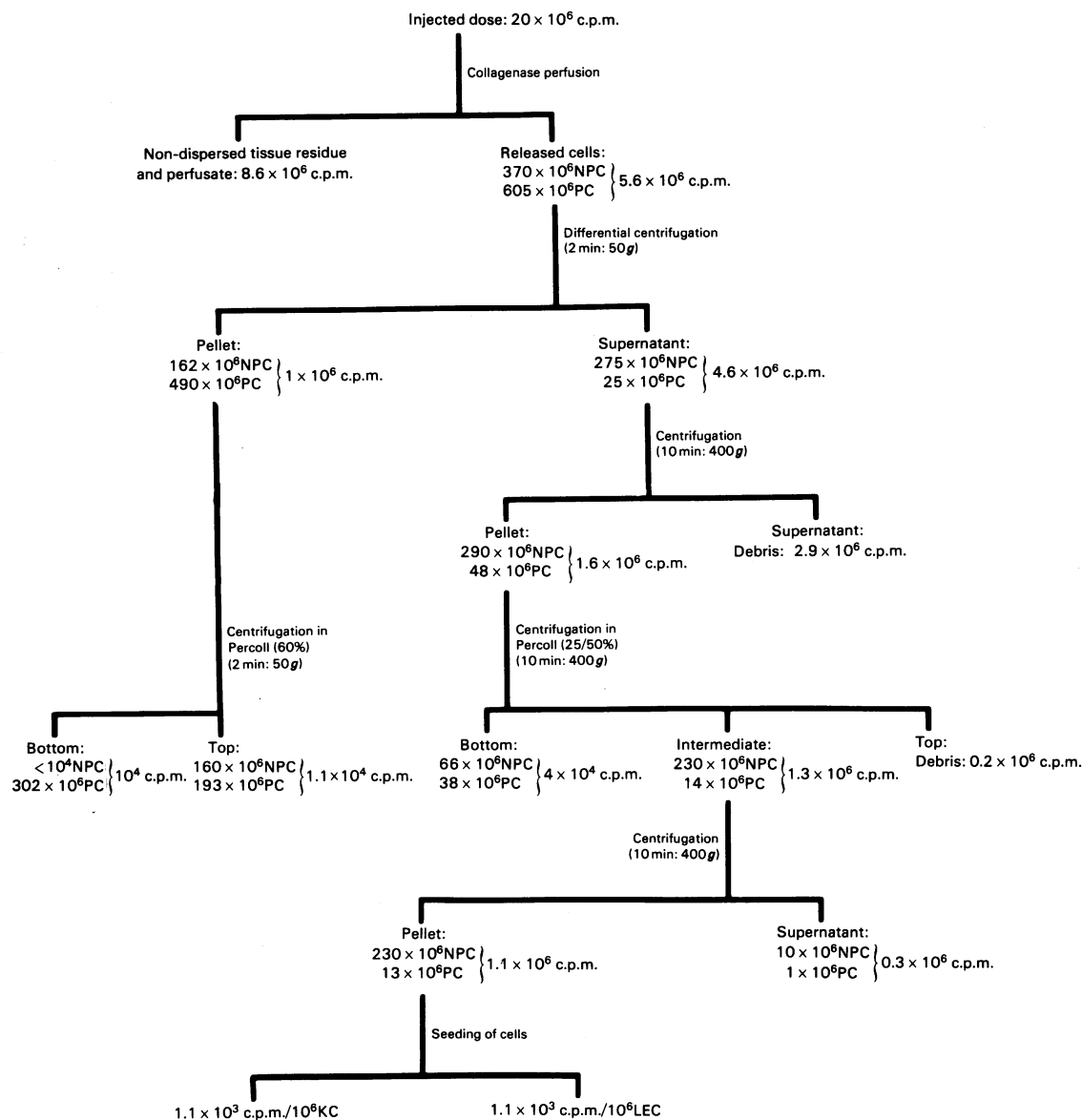
Table 2. Interaction of  $^{125}\text{I}$ - $\alpha 1$  with cultivated Kupffer and liver endothelial cells  
Cultures of Kupffer cells ( $70000\text{ cells/cm}^2$ ) and liver endothelial cells ( $200000\text{ cells/cm}^2$ ) grown in  $9.6\text{ cm}^2$  dishes, media conditioned for 30 min with such cultures, or fresh medium containing bacterial collagenase, were incubated with the ligands at  $37^\circ\text{C}$  for 30 min. The values given (means for duplicate experiments) represent the percentage bound or degraded of added amounts, which were  $0.15\text{ pmol}$  of  $^{125}\text{I}$ - $\alpha 1$  ( $3 \times 10^5$  c.p.m.) or  $^{125}\text{I}$ -fibronectin ( $2 \times 10^5$  c.p.m.) per dish.

Incubation protocol	Percentage of added amount		
	Cell-associated $^{125}\text{I}$ - $\alpha 1$	Degraded	
		$^{125}\text{I}$ - $\alpha 1$	$^{125}\text{I}$ -fibronectin
Liver endothelial cells	8.8	29.5	n.d.*
+ Fibronectin ( $300\text{ }\mu\text{g/ml}$ )	6.7	33.3	n.d.
Kupffer cells	0.3	70.2	n.d.
+ Fibronectin ( $300\text{ }\mu\text{g/ml}$ )	0.3	71.0	n.d.
Spent medium from:			
liver endothelial cells		8.5	7.5
Kupffer cells		43.0	5.9
Fresh medium		7.1	4.9
+ Advance Biofacture collagenase†		94.5	5.7
+ Sigma collagenase‡		100	6.0

\* n.d., not determined.

† Proteinase-free, 2600 units/ml.

‡ Type V (*Clostridium histolyticum*);  $0.1\text{ mg/ml}$ .



Scheme 1. Identification of the liver cells responsible for the clearance of circulating  $^{125}\text{I}-\alpha 1$

Flow scheme showing the radioactivity and the yield of cells at the various steps of the isolation of liver cells after the intravenous administration of  $9 \mu\text{g}$  of  $^{125}\text{I}-\alpha 1$ . Note that the preparation of pure hepatocytes (see the middle of the Scheme) obtained by centrifugation through 60% (w/v) Percoll, contained only about 30 c.p.m. per  $10^6$  cells. In contrast, purified Kupffer cells and liver endothelial cells (bottom of the Scheme) both contained  $1.1 \times 10^3$  c.p.m. per  $10^6$  cells. The procedures for the isolation and identification of the various cell types were as described in the Materials and methods section. Abbreviations used: PC, parenchymal cells (hepatocytes); NPC, non-parenchymal cells; KC, Kupffer cells; LEC, liver endothelial cells.

liver endothelial cells, and fresh medium, were incubated with the ligand. Incubation of  $^{125}\text{I}-\alpha 1$  in fresh medium, either with the purified bacterial collagenase from Advance Biofacture, or with the more crude bacterial collagenase preparation from Sigma served as positive controls for the specific

degradation of the ligand. As shown in Table 2, spent media from the Kupffer-cell cultures degraded  $^{125}\text{I}-\alpha 1$  but not  $^{125}\text{I}$ -fibronectin. The same result was obtained with the two collagenases. In contrast, spent media from the liver endothelial-cell cultures were as inactive as fresh medium.

The kinetics of uptake and degradation at 37°C of  $^{125}\text{I}$ - $\alpha 1$  by liver endothelial-cell cultures is shown in Fig. 2. The cell-associated pool of label increased during the first 1 h of incubation. Owing to release of degradation products, and because of depletion of ligand in the medium, the cell-associated radioactivity decreased after the initial 1 h of incubation.

*Specificity of binding and uptake of  $^{125}\text{I}$ - $\alpha 1$  by liver endothelial cells*

$^{125}\text{I}$ - $\alpha 1$  that had associated with the liver endothelial cells at 4°C could be quantitatively removed from the cells by treatment with bacterial collagenase (results not shown), indicating that endocytosis did not occur at this temperature.

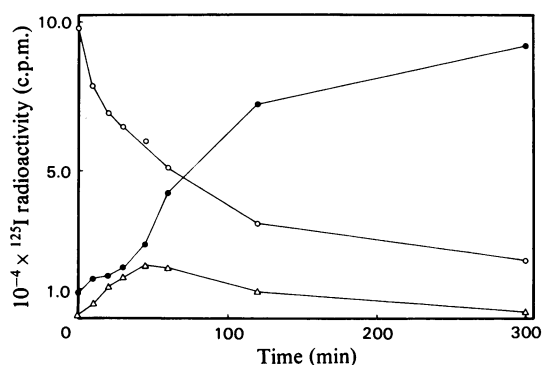


Fig. 2. Kinetics of uptake and degradation of  $^{125}\text{I}$ - $\alpha 1$  by cultured liver endothelial cells

Cultures of liver endothelial cells ( $2\text{cm}^2$  growth areas) were incubated with  $^{125}\text{I}$ - $\alpha 1$  ( $0.2\text{ nM}$ ) at 37°C. After various periods of time the accumulation of label in the cell layer ( $\Delta$ ), and the content in the medium of undegraded ( $\circ$ ) and degraded ( $\bullet$ ) ligand were determined as described in the Materials and methods section. The points represent the mean results for two experiments.

However, transfer of the cultures to 37°C and a medium without  $^{125}\text{I}$ - $\alpha 1$ , subsequent to binding of  $^{125}\text{I}$ - $\alpha 1$  at 4°C, led to degradation of the ligand (Fig. 3). This finding indicates that binding at 4°C occurred via the same mechanism that led to endocytosis at 37°C. In order to investigate the specificity of the binding of  $^{125}\text{I}$ - $\alpha 1$  by the liver endothelial cells, competition experiments were carried out with various compounds. As shown in Table 3, unlabelled  $\alpha 1$  inhibited the binding of the

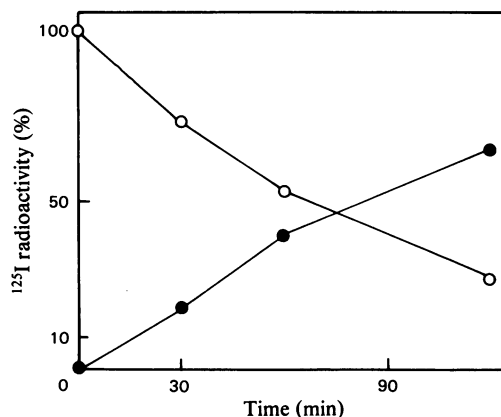


Fig. 3. Degradation at 37°C of  $^{125}\text{I}$ - $\alpha 1$  bound to cultured liver endothelial cells at 4°C

Cultures of liver endothelial cells ( $9.6\text{cm}^2$  growth areas) were incubated with  $^{125}\text{I}$ - $\alpha 1$  ( $0.75\text{ nM}$ ;  $10^5\text{ c.p.m.}$ ) at 4°C for 12 h. After washing of the cell layers to remove unbound ligand, the cultures were transferred to 37°C and assayed for radioactivity in the cell layer ( $\circ$ ) and in the medium ( $\bullet$ ). The amount of radioactivity bound to the cell layer at the time of transfer to 37°C ( $18 \times 10^3\text{ c.p.m.}$ ) was taken as 100%. All label released to the medium was acid-soluble. See the Materials and methods section for assay procedures. The values represent the means for two experiments.

Table 3. Specificity of binding of  $\alpha 1$  to liver endothelial cells

Monolayers of liver endothelial cells ( $9.6\text{cm}^2$  cultures) were incubated with a trace concentration of  $^{125}\text{I}$ - $\alpha 1$  ( $0.25\text{ nM}$ ) along with the test substances listed in the Table. The amounts of  $^{125}\text{I}$ - $\alpha 1$  that were associated with the cells in the absence of test substance at 37°C and 4°C respectively were taken as 100%. The values represent the means for duplicate experiments.

Substance tested	Concn. ( $\mu\text{g/ml}$ )	Cell-associated radioactivity (%)	
		Temperature ... 37°C Incubation time ... 30 min	4°C 12 h
None		100	100
$\alpha 1$	100	4	4
Hyaluronic acid	100	90	106
Chondroitin sulphate	100	103	122
Ovalbumin	100	113	102
Fibronectin	300	90	100
Anti-fibronectin F(ab') <sub>2</sub>	500	94	n.d.

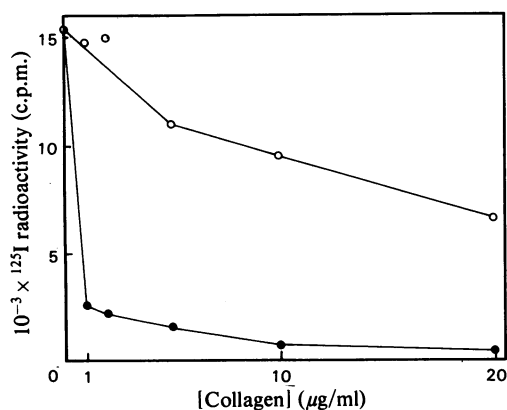


Fig. 4. Different affinities of the liver endothelial-cell  $\alpha 1$ -receptor to native and denatured collagen

Cultures of liver endothelial cells ( $2\text{cm}^2$  growth areas) were incubated with  $^{125}\text{I}$ - $\alpha 1$  ( $0.25\text{ nM}$ ) at  $37^\circ\text{C}$  in the presence of increasing amounts of unlabelled native (○) or heat-denatured (●) neutral salt-soluble collagen. After 60 min the cell layer was analysed for radioactivity as described in the Materials and methods section. The values represent means for two experiments.

label by more than 96%, whereas hyaluronic acid, chondroitin sulphate and ovalbumin had inhibitory effect. Interestingly, heat-denatured collagen inhibited far more effectively the binding of  $^{125}\text{I}$ - $\alpha 1$  to the liver endothelial cells than did native collagen (Fig. 4). Thus a 13-fold excess of heat-denatured collagen ( $1\text{ }\mu\text{g/ml}$ ) inhibited the binding of  $^{125}\text{I}$ - $\alpha 1$  by 80%, whereas as much as a 260-fold excess of native collagen ( $20\text{ }\mu\text{g/ml}$ ) gave only a 50% reduction in binding.

#### Mechanisms of uptake and degradation of $\alpha 1$ by liver endothelial cells

Incubation of liver endothelial cells with fluorescein-labelled heat-denatured neutral-salt-soluble collagen resulted in accumulation of the dye in intracellular vesicles (Fig. 5), suggesting that the ligand was indeed internalized by the cells. Additional evidence of endocytosis was obtained by employing (1) the lysosomotropic agents ammonia, chloroquine and dansylcadaverine, (2) the carboxylic ionophore monensin and (3) the microfilament depolymerizer cytochalasin B. These compounds, which are known to block one or several steps of the endocytic machinery, markedly inhibited the accumulation of  $^{125}\text{I}$ - $\alpha 1$  into the liver endothelial cells (Fig. 6). In contrast, the enzyme inhibitor leupeptin caused excess intracellular accumulation of the ligand (Fig. 6). Although this drug inhibited the degradation of  $^{125}\text{I}$ - $\alpha 1$  by 30–40% (Fig. 7), the sum of cell-associated plus

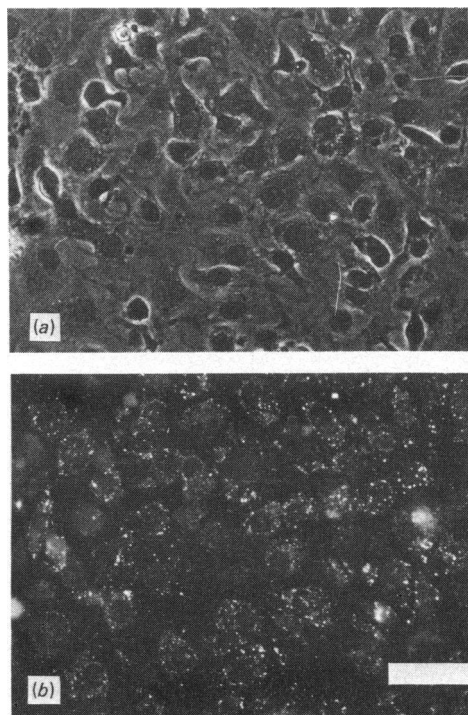


Fig. 5. Uptake of fluorescein-labelled collagen by liver endothelial cells

Cultivated liver endothelial cells were incubated for 1 h at  $37^\circ\text{C}$  with heat-denatured neutral-salt-soluble collagen ( $0.1\text{ mg/ml}$ ) that had been conjugated to fluorescein isothiocyanate. The cultures were then washed and prepared for optical-microscopic examination with phase-contrast (a) and fluorescence (b) optics. As revealed by the numerous fluorescent perinuclear vesicles (b), the dye was taken up by virtually all the cells. The wide horizontal bar represents  $40\text{ }\mu\text{m}$ .

degraded labelled material was the same in the leupeptin-treated cultures as in the untreated control cultures. This result indicates that leupeptin selectively inhibits the degradation of  $\alpha 1$  collagen, although the extent of uptake is unaffected.

#### Discussion

##### Fate of circulating $^{125}\text{I}$ - $\alpha 1$

Monomeric  $^{125}\text{I}$ - $\alpha 1$  collagen type I survived only a few minutes in the circulation, reflecting an efficient clearance of this protein. The lag period of approx. 10 min observed before the appearance in the circulation of low- $M_r$   $^{125}\text{I}$ -labelled degradation products is compatible with intralysosomal rather than extracellular degradation.

The findings that the Kupffer cells and the liver

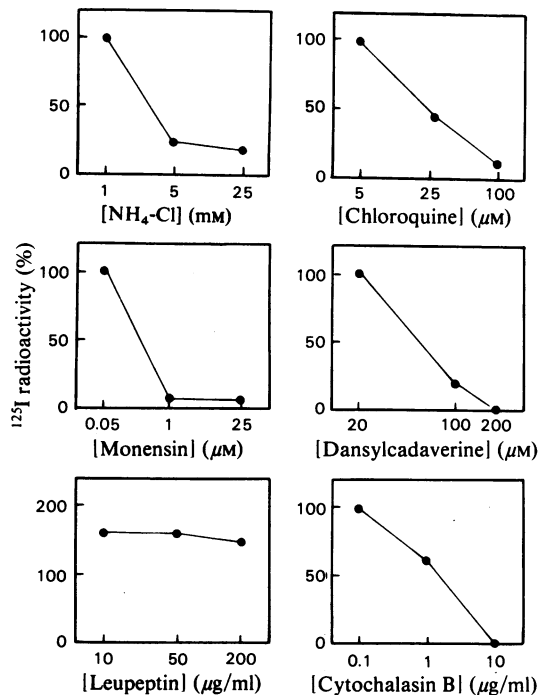


Fig. 6. Effect of endocytic and lysosomal inhibitors on the uptake of <sup>125</sup>I-α1 by cultivated liver endothelial cells. Cultures of liver endothelial cells (2cm<sup>2</sup> growth area) were pre-incubated for 30 min at 37°C in the presence of six types of inhibitors of endocytic and lysosomal function. <sup>125</sup>I-α1 (0.25 nM) was then added and, after an additional 60 min of incubation, the cell layers were analysed for radioactivity. The amount of ligand taken up in the absence of inhibitors was considered to be 100% uptake. Note that because leupeptin increased the cellular content of label to more than 100%, the ordinate of this panel was extended to 200%. The values represent the means for two experiments.

endothelial cells *in vivo* took up equal amounts of the ligand per cell, along with the fact that the liver endothelial cells are about three times more abundant than the Kupffer cells (Knook & Sleyster, 1976), imply that the liver endothelial cells constitute the main site of sequestration of circulating α1. By using density separation in Percoll to obtain highly purified preparations of hepatocytes (Scheme 1), these cells were shown not to contribute to the clearance of this collagen species.

#### Interaction of <sup>125</sup>I-α1 collagen with isolated liver cells

The amount of label taken up per cell was about 10 times higher in the liver endothelial cells than in the Kupffer cells (Table 2). Spent media from

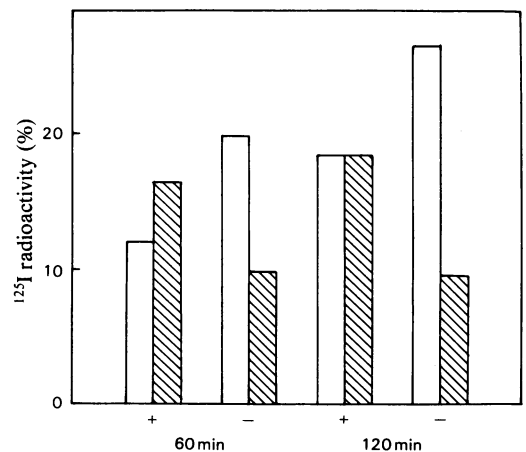


Fig. 7. Effect of leupeptin on the uptake and degradation of <sup>125</sup>I-α1 by cultivated liver endothelial cells.

Cultures of liver endothelial cells (2cm<sup>2</sup> growth area) were pre-incubated for 30 min at 37°C in the presence of leupeptin (10 μg/ml). <sup>125</sup>I-α1 (0.5 pmol) was then added and, after an additional 60 min or 120 min of incubation, the cultures were analysed for degraded material (□) and cell-associated radioactivity (▨) as described in the Materials and methods section. Each bar represents the mean of two experiments. +, Leupeptin present in the cultures; -, controls (leupeptin-free cultures). The ordinate shows radioactivity expressed as a percentage of the added dose (110000 c.p.m.).

Kupffer-cell cultures contained activity that degraded <sup>125</sup>I-α1 but not <sup>125</sup>I-fibronectin, indicating that these cells secreted (an) enzyme(s) that specifically attacked the α1 molecules. This finding agrees with a previous report describing secretion of collagenolytic activity by Kupffer cells (Fujiwara *et al.*, 1973). Conceivably, even if the Kupffer cells express receptors for endocytosis of α1, the α1-degrading activity present in the medium might have broken down the ligand to the extent that it was no longer recognized by the receptors. The finding that the Kupffer cells *in vivo* but not *in vitro* took up <sup>125</sup>I-α1 may be explained along the lines that collagenolytic activity is either inhibited *in vivo* by specific inhibitors in the blood (Harper, 1980) or, alternatively, the Kupffer cells do not normally secrete this enzyme *in situ*.

The opsonic effect of fibronectin on the uptake of soluble as well as particulate gelatin by cells of the mononuclear phagocytic system has been well documented (Dessau *et al.*, 1978; Saba *et al.*, 1978). However, since the addition of fibronectin to the liver endothelial cells did not stimulate the uptake of <sup>125</sup>I-α1, and further, since F(ab')<sub>2</sub> fragments of anti-fibronectin antibodies had no inhibitory effect, it is concluded that the uptake of α1 collagen

by the liver endothelial cells does not involve fibronectin.

*Specificity of binding and uptake of  $^{125}\text{I}$ - $\alpha 1$  by liver endothelial cells*

The finding that an excess of unlabelled  $\alpha 1$  collagen blocked the binding and uptake of trace amounts of  $^{125}\text{I}$ - $\alpha 1$  suggests that this collagen species interacts with liver endothelial cells via a specific binding site. Further, heat-denatured collagen competed far more efficiently for binding to this receptor than did native collagen, indicating that the  $\alpha 1$ -receptor specifically recognizes non-helical collagen chains. In view of the fact that the cleavage products from the attack on native collagen by vertebrate collagenase readily denature at 37°C (Sakai & Gross, 1967), the  $\alpha 1$ -binding site on the liver endothelial cells could serve an important physiological function by rapidly removing denatured collagen peptides from the circulation. The connective-tissue components hyaluronic acid and chondroitin sulphate, as well as glycoproteins with terminal mannose and *N*-acetylglucosamine residues, have been previously found to be endocytosed by the liver endothelial cells via specific receptors (Hubbard *et al.*, 1979; Smedsrød *et al.*, 1984). Since none of these substances interfered with the binding and uptake of  $\alpha 1$ , it is concluded that the  $\alpha 1$ -binding site is a distinct entity on the liver endothelial cells.

*Mechanisms of uptake and degradation of  $^{125}\text{I}$ - $\alpha 1$  by liver endothelial cells*

The following lines of evidence suggest that the degradation of  $^{125}\text{I}$  by liver endothelial cells is preceded by endocytosis of the ligand. (1) Spent media from cultures of liver endothelial cells did not degrade  $\alpha 1$ . (2) A lag period between uptake of  $^{125}\text{I}$ - $\alpha 1$  and release of low- $M_r$   $^{125}\text{I}$ -containing degradation products signifies intralysosomal degradation *in vivo* and *in vitro*. (3) Incubation of liver endothelial cells with fluorescein-labelled gelatin resulted in accumulation of the dye in vesicles of the cells. (4) Chloroquine, cytochalasin B, dansylcadaverine, monensin, and ammonia, which block one or more steps of the endocytic and lysosomal machineries (Marsh, 1984; Wessels *et al.*, 1971), markedly inhibited the association of  $^{125}\text{I}$ - $\alpha 1$  with the liver endothelial cells. (5) Leupeptin, a bacterial oligopeptide that specifically inhibits thiol proteinases (Umezawa, 1977), inhibited the degradation of  $^{125}\text{I}$ - $\alpha 1$  by 30–40%, without affecting the extent of uptake of the ligand. The latter observation, along with the reported finding that leupeptin-inhibitable cathepsin B<sub>1</sub> and collagenolytic cathepsin are essential for the lysosomal degradation of collagen (Burleigh *et al.*, 1974;

Etherington, 1976), strongly suggest that the degradation of  $\alpha 1$  takes place intralysosomally.

*Physiological relevance of the  $\alpha 1$ -receptor on liver endothelial cells*

The finding that monomeric  $\alpha 1(\text{I})$  collagen is cleared from the circulation mainly via receptor-mediated endocytosis in the liver endothelial cells suggests that these cells may play an important role in the turnover of soluble collagen peptides that are released to the circulation under physiological as well as under pathophysiological conditions. Recently it was also shown that the liver endothelial cells constitute the main site of catabolism of circulating hyaluronic acid and chondroitin sulphate (Smedsrød *et al.*, 1984, 1985). It thus appears that the liver endothelial cells play a key role in the clearance and catabolism of the major connective-tissue macromolecules.

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